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Research Article

GC-MS Analysis and Antibacterial Activity of Ethanolic and Water Extracts of Malaysian *Heterotrigona itama* Propolis Against Selected Human Pathogenic Bacteria

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ABSTRACT

Despite the growing interest in the therapeutic potential of propolis, limited attention has been paid to the chemical composition and biological activity of water extract propolis produced by Malaysian stingless bees. Thus, this study aimed to determine the phytochemical composition and antibacterial potential of ethanolic extract propolis (EEP) and water extract propolis (WEP) of the stingless bee species, *Heterotrigona itama* against ten pathogenic bacteria. The phytochemical analysis of the extracts was carried out using the gas chromatography-mass spectrometry (GC-MS) technique. The antibacterial activity was determined using the disc-diffusion, minimum inhibition concentration (MIC), and minimum bactericidal concentration (MBC) methods. The GC-MS analysis of EEP exhibited four volatile compounds including hexamethylcyclotrisiloxane, 2-hydroxy-2-cyclopenten-1-one, 2-coumaranone, and diethyl bis(trimethylsilyl) ester silicic acid. However, only two compounds were identified in WEP, consisting of 2-(acetoxymethyl)-3-(methoxycarbonyl)biphenylene, and hexamethylcyclotrisiloxane. EEP showed the highest antibacterial activity against all Gram-positive bacteria (*Bacillus subtilis*, *Enterococcus faecalis*, *Enterococcus faecium*, *Micrococcus luteus*, *Staphylococcus aureus*, *Streptococcus mutans*) with values of the inhibition zones ranging from 7 to 10 mm. However, both extracts showed no antibacterial activity against Gram-negative bacteria, except WEP, which displayed an inhibition zone of 9.33 ± 1.53 mm against *Escherichia coli*. Meanwhile, EEP showed the lowest MIC and MBC values against *M. luteus* at 70 and 280 µg/mL, respectively. The results revealed the presence of several volatile compounds in the EEP of *H. itama* which could contribute to its antibacterial activity, particularly against Gram-positive bacteria.

Key words: Antibacterial, disc diffusion, GC-MS, *Heterotrigona itama*, minimum inhibitory concentration, stingless bee propolis

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INTRODUCTION

Stingless bees are a diverse group of eusocial bees that play an important role in the pollination of native plants, particularly in tropical and subtropical regions of the world (Li *et al*., 2021). More than 500 species of stingless bees have been documented worldwide, with the greatest diversity found in Asia, Africa, Australia, and Latin America (Souza *et al*., 2021). These regions represent significant areas for investigating the ecological and economic importance of these distinctive pollinators. In Malaysia, there are 45 identified species of stingless bees, locally referred to as "kelulut", with *Heterotrigona itama* being the most dominant and prevalent species in most ecosystems (Fahimee *et al*., 2021; Jaapar *et al*., 2016). It is typically characterized by its black coloration and grey wings, earning it the nickname of the "black jet" species (Azmi *et al*., 2019). *H. itama* is widely distributed throughout the Malay Archipelago encompassing Peninsular Malaysia, Malaysian Borneo, Singapore, Southern Thailand, and the islands of Java, Kalimantan, and Sumatra. The insect plays a critical role as a pollinator for diverse crops, which are essential components of the economy (Khalifa *et al*., 2021; Azmi *et al*., 2022). In Malaysia, *H. itama* is extensively domesticated in the meliponiculture industry, mainly for the production of honey, and propolis (Ivorra *et al*., 2020).

Propolis is a resinous substance produced by bees from the selective collection of plant buds or exudates (Kustiawan *et al*., 2023). It is used as a sealant for beehives and acts as a protective barrier against foreign invaders as well as against weathering of the hive (Salleh *et al*., 2022). Propolis hardens the wall of hives and contributes to an aseptic internal environment (Pasupuleti *et al*., 2017). The antimicrobial property of propolis plays a significant role in the beehive's defense against bacteria, viruses, and other pathogenic microorganisms that may invade the colony (Touzani *et al*., 2019). Additionally, propolis has been used in traditional medicine for centuries and is highly regarded for its numerous health benefits, including antioxidant, antibacterial, antiviral, antifungal, anti-inflammatory, and immunomodulatory activities (Popova *et al*., 2021). In recent years, there has been a surge of interest in the health-promoting properties of propolis, leading to extensive research on its biological applications (Al-Hatamleh *et al*., 2020). Propolis produced by *Apis mellifera*, which is the most common honeybee species in Europe, Africa, and the Middle East, has been dominating the field of propolis research and commercialization (Anjum *et al*., 2019). However, recent research suggests that propolis produced by stingless bees (Meliponinae) has greater nutritional and medicinal benefits in comparison to honeybee propolis (Al-Hatamleh *et al*., 2020; Salleh *et al*., 2022). The unique foraging behavior of stingless bees, which involves visiting a diverse range of plant species to collect resin, pollen, nectar, and other plant-derived materials, may contribute to this greater value (Asma *et al*., 2019).

Stingless bee propolis represents a vast repository of chemical diversity, containing a wide range of phytochemical compounds such as polyphenols, amino acids, steroids, terpenoids, and inorganic compounds (Kasote *et al*., 2019; Popova *et al*., 2021). The variability in its chemical composition and consequent biological activities depends on various factors, such as geographical location, seasonality, botanical origin, bee species, and extraction solvents (Popova *et al*., 2021). Typically, propolis extracts are obtained through conventional methods using ethanol as the solvent to attain a low-wax propolis extract while retaining its rich bioactive compounds, particularly polyphenols (Devequi Nunes *et al*., 2018). However, several disadvantages have been associated with propolis ethanolic extract such as strong residual flavor, and adverse reactions or intolerance to alcohol which limit its application in the cosmetics, pharmaceutical, and food industries (Kubiliene *et al*., 2015; Salleh *et al*., 2021). In contrast, water extract propolis has the advantages of being safe for human health and the environment, and biocompatible for use in the pharmaceutical and healthcare sectors (Kubiliene *et al*., 2018). Hence, this study aimed to determine the phytochemical constituents and antibacterial properties of ethanolic and water extracts of *H. itama* propolis against several human pathogenic bacteria.

MATERIALS AND METHODS

Propolis collection

Propolis produced by stingless bees *H. itama* was collected from a local apiary, Belantara SR Enterprise (N 3° 40' 42.1818" E 10° 31' 14.5416"), Hulu Bernam, Selangor in December 2021. The colonies were housed in wooden nest boxes under the shade with predominant vegetation consisting of *Lamiaceae*, *Myrtaceae*, *Rubiaceae*, *Fabaceae*, *Lythraceae*, *Scrophulariaceae*, *Elaeocarpaceae*, *Sapindaceae*, *Moraceae*, and *Anacardiaceae*. The stingless bee species was identified by an entomologist from the Centre for Insect Systematics (CIS), Universiti Kebangsaan Malaysia with the accession number CIS-TRI-2022-02 (Adli *et al*., 2022). The propolis sample was collected by scraping the inside of the hives using a clean stainless-steel spatula. Crude propolis was cleaned, air-dried, and stored in a polyethylene bag at -20 °C until processing.

Preparation of Ethanolic Extract Propolis (EEP)

The EEP was prepared according to the method described by Pobiega *et al*. (2019) with slight modifications. Briefly, 10 g of the propolis was ground into a fine powder and mixed with 100 mL of 70% ethanol in a 1:10 (w/v) ratio. Then, the sample was incubated at 25 °C and continuously shaken at 250 rpm for 48 h. The suspension was filtered using Whatman No. 1 filter paper (Millipore, USA) and the filtrate was evaporated under reduced pressure (Rotavapor R-215, Büchi, Switzerland) at 40 °C. The concentrated extract was centrifuged at 3,900 × *g* for 10 min to eliminate wax. Next, the extract was freeze-dried (SCANVAC Coolsafe™, model 110-4, Denmark) and kept at 4 °C in a dark container until further use.

Preparation of Water Extract Propolis (WEP)

The WEP was obtained following the procedure described by Al-Ani *et al*. (2018) with slight modifications. An amount of 10 g of propolis was ground into a fine powder and dissolved in 50 mL of distilled water at a 1:5 ratio (w/v). Next, the sample was heated on a hot plate with constant stirring at 60 °C for 7 h. The suspension was filtered using Whatman No. 1 filter paper (Millipore, USA), and the filtrate was centrifuged at 28,000 × *g* for 30 min. The supernatant was concentrated under reduced pressure to produce the extract, freeze-dried, and kept at 4 °C in a dark container until use.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The phytochemical analysis of the extracts was carried out using Agilent G-7890A (Agilent Technologies Inc., California, USA) GC-MS system equipped with a 5975C inert MSD with the triple-axis detector, fitted with a Hewlett Packard HP-5MS silica capillary column (30 m × 0.25 mm internal diameter, 0.25 µm film thickness). The instrument was operated in electron impact mode with 70 eV of ionization energy, and injector temperature at 250 °C. Helium gas was used as the carrier gas at a flow rate of 1 mL/min. A volume of 1 µL of the sample was injected automatically in a 1:1 split mode. The oven temperature was initially programmed at 50 °C (hold for 3 min) and then increased to 300 °C at 20 °C/min and hold for 2 min. The obtained data were processed and analyzed with the Agilent ChemStation software. The identification of compounds from the spectral data was based on the available mass spectral records from the National Institute of Standards and Technology (NIST) 2008 library. The results were analyzed using MS full scan mode (*m/z* 50 - 750).

Bacterial strains and growth conditions

A total of 10 bacterial strains were used in this study, which includes six Gram-positive bacteria (*Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium* ATCC 51858, *Micrococcus luteus* ATCC 48732, *Staphylococcus aureus* ATCC 25923 and *Streptococcus mutans* clinical isolate) and four Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 9027 and *Salmonella typhimurium* ATCC 14028). The stock cultures were maintained in nutrient agar (NA) slant at 4 °C. Working cultures were prepared by inoculating a loopful of each test microorganism in 10 mL of Mueller-Hinton Broth (MHB) from NA slants and incubated at 37 °C for 18-24 h. The density of overnight cultured was standardized to 0.5 McFarland solutions (1×10^8 CFU/mL) before being used for the antibacterial assay.

Sample preparation

A stock solution of EEP and WEP was prepared by dissolving 0.1 g of dry extract in 100% dimethyl sulfoxide (DMSO) to produce the final concentration of 100 mg/mL. The working solutions were prepared by diluting the stock solution with sterile distilled water until the concentration reached 10 mg/mL.

Disc diffusion assay

Antibacterial susceptibility test of propolis extracts was performed by disc diffusion method against bacterial strains following Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2018). Petri dishes with Mueller-Hinton Agar (MHA) were seeded with 0.5 McFarland bacterial suspension using a sterile cotton swab to obtain lawn culture. Each sterile blank disc (6 mm in diameter) was impregnated with 30 µL of the extracts and allowed to dry. Subsequently, the discs were placed on the surfaces of MHA plates that were pre-inoculated with bacterial cultures and incubated at 37 °C for 24 h. All tests were carried out in triplicates. Standard antibiotic discs were selected according to the sensitivity of the bacteria tested. Gentamicin (10 µg/disc) and ceftriaxone (30 µg/disc) were used as positive controls, while 10% DMSO served as a negative control. The results were obtained by measuring the diameter of the inhibition zone around the discs. Antibacterial activity was expressed as the mean zone of inhibition diameters (mm) produced by the extracts.

Minimum Inhibitory Concentration (MIC)

The MIC of the extracts was determined using the modified resazurin broth microdilution method (Sarker *et al*., 2007). MIC was evaluated on bacterial strains that showed sensitivity to the extracts in the disc diffusion assay. A two-fold serial dilution of each extract, with starting concentration of 100 mg/mL was prepared using cation-adjusted MHB as a diluent, resulting in concentrations of 9000 to 35.2 µg/mL. A 50 µL of bacterial suspension (1×10^6 CFU/mL) was then dispensed using a micropipette into each of the above-loaded wells. All tests were carried out in triplicate using a sterile 96-well microtiter plate. Each microplate consisted of a set of controls: sterility control (broth only), growth control (broth and bacteria), and negative control (1% DMSO). Gentamicin and ceftriaxone were used as positive controls with an initial concentration of 1 mg/mL. The plates were incubated at 37 °C for 24 h. After incubation, 10 µL of 0.01% resazurin indicator solution (Sigma Aldrich, US) was added to each well and incubated for another 2 h at 37 °C under anaerobic conditions. Color changes were observed and recorded. The lowest concentration of extracts with no color change (blue resazurin color remained unchanged) was taken as the MIC. This experiment was conducted in triplicate.

Minimum Bactericidal Concentration (MBC)

To determine the minimum bactericidal concentration (MBC), 10 µL of the culture from each well of the micro broth assay was sub-cultured on MHA plates after 24 h of incubation. MHA plates were further incubated for 24 h. The lowest concentration of extracts that exhibited no bacterial growth was deliberated as the MBC values. The experiment was repeated in triplicate for each bacterial strain.

Statistical Analysis

Values were expressed as mean ± standard deviation (SD). The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test using the SPSS program for Windows, version 17.0 (SPSS, IL, USA). Differences between groups were considered significant at p-value< 0.05.

RESULTS

GC-MS analysis

The GC-MS analysis of EEP and WEP revealed the presence of several volatile compounds as listed in Table 1. Four compounds were identified in EEP consisting of hexamethylcyclotrisiloxane (9.62%), 2-hydroxy-2-cyclopenten-1-one (4.68%), 2-coumaranone (4.25%), and diethyl bis(trimethylsilyl) ester silicic acid (0.05%). Meanwhile, the two compounds identified in WEP were 2-(acetoxymethyl)-3-(methoxycarbonyl)biphenylene (2.39%), and hexamethylcyclotrisiloxane (5.94%).

Disc diffusion assay

Table 2 shows the mean diameter of inhibition zones produced by each extract on tested bacteria. The EEP demonstrated antibacterial activities against all tested Gram-positive bacteria, with inhibition zones ranging from 7.00 to 10.00 mm. EEP exhibited the greatest zone of inhibition at 10.00 ± 0.58 mm against *M. luteus*. Although the antibacterial activity of WEP was significantly lower than EEP, it still exhibited inhibition against *B. subtilis* and *S. mutans*, with an inhibition zone of 6.00 ± 0.00 and 6.33 ± 0.29 mm, respectively.

However, none of the extracts showed activity against all tested Gram-negative bacteria, except WEP with an inhibition zone of 9.33 ± 1.53 mm against *E. coli*. The positive controls, gentamicin, and ceftriaxone, showed the greatest antibacterial activities against all tested bacteria with significant inhibition zones ranging from 10.67 to 37.33 mm, and 12.00 to 41.67 mm, respectively.

Table 1. Chemical compounds in ethanolic extract propolis (EEP) and water extract propolis (WEP) of *H. itama*

Compound	Compound group	Retention time (min)	Area (%)	
			EEP	WEP
Hexamethylcyclotrisiloxane	Organosilicon	14.718	9.62	2.39
2-(Acetoxymethyl)-3-(methoxycarbonyl) biphenylene	Aromatic compound	15.040	-	5.94
2-hydroxy-2-cyclopentene-1-one	Carbonyl group	5.111	4.68	-
2-Coumaranone	Ketone	7.782	4.25	-
Diethyl bis(trimethylsilyl) ester silicic acid	Silicon ester	14.058	0.05	-

Table 2. Zone of inhibition of ethanolic extract propolis (EEP) and water extract propolis (WEP) against selected pathogenic bacteria

Bacteria	Inhibition zones (mm)			
	EEP	WEP	Gentamicin[1]	Ceftriaxone[2]
Gram-positive bacteria				
B. subtilis	8.67 ± 0.58^{b}	6.00 ± 0.00^{a}	21.33 ± 0.58^{c}	23.67 ± 0.58^{c}
E. faecalis	7.00 ± 0.00^{a}	-	11.00 ± 0.00^{b}	15.33 ± 3.06^{c}
E. faecium	7.00 ± 0.00^{a}	-	10.67 ± 0.58^{b}	16.00 ± 0.00^{c}
M. luteus	10.00 ± 0.58^{a}	-	37.33 ± 1.53^{b}	41.67 ± 1.15^{c}
S. aureus	8.00 ± 0.00^{a}	-	23.67 ± 1.15^{b}	22.00 ± 1.73^{b}
S. mutans	8.00 ± 1.00^{b}	6.33 ± 0.29^{a}	20.00 ± 0.00^{d}	12.00 ± 3.46^{c}
Gram-negative bacteria				
E. coli	-	9.33 ± 1.53^{a}	21.33 ± 0.58^{b}	29.67 ± 0.58^{c}
K. pneumoniae	-	-	11.33 ± 0.58^{a}	16.00 ± 1.00^{b}
P. aeruginosa	-	-	22.00 ± 0.00^{a}	20.50 ± 2.18^{a}
S. typhimurium	-	-	17.67 ± 0.58^{a}	29.67 ± 0.58^{b}

Values are expressed as mean ± standard deviation of three replicates. The mean in the same row with different superscripts is significant at $p<0.05$. [1]Gentamicin at 10 µg/disc; [2]Ceftriaxone at 30 µg/disc; "-": no inhibition zones observed.

Minimum Inhibitory Concentration (MIC)

The effectiveness of the extracts on tested bacterial strains was determined by measuring the minimum inhibitory concentration (MIC) (Table 3). The EEP showed MIC values of 2250 µg/mL against *E. faecalis* and 4500 µg/mL against *E. faecium*, *S. aureus,* and *S. mutans*. Furthermore, EEP exhibited the lowest MIC value of 70 µg/mL against *M. luteus*. The WEP showed antibacterial activity against *S. mutans* with a MIC of 9000 µg/mL. However, no inhibition was observed against *B. subtilis* and *E. coli* for EEP and WEP at a concentration of 9000 µg/mL.

Table 3. The minimum inhibitory concentration (MIC) of ethanolic extract propolis (EEP) and water extract propolis (WEP) against selected pathogenic bacteria

Bacteria	Concentration (µg/mL)			
	EEP	WEP	Gentamicin[1]	Ceftriaxone[2]
Gram-positive bacteria				
B. subtilis	>9000.00 ± 0.00	>9000.00 ± 0.00	1.56 ± 0.00	0.78 ± 0.00
E. faecalis	2250.00 ± 0.00	-	2250.00 ± 0.00	2250.00 ± 0.00
E. faecium	4500.00 ± 0.00	-	4500.00 ± 0.00	4500.00 ± 0.00
M. luteus	70.00 ± 0.00	-	70.00 ± 0.00	70.00 ± 0.00
S. aureus	4500.00 ± 0.00	-	4500.00 ± 0.00	4500.00 ± 0.00
S. mutans	4500.00 ± 0.00	4500.00 ± 0.00	4500.00 ± 0.00	4500.00 ± 0.00
Gram-negative bacteria				
E. coli	-	>9000.00 ± 0.00	0.39 ± 0.00	0.10 ± 0.00
K. pneumoniae	-	-	12.50 ± 0.00	50.00 ± 0.00
P. aeruginosa	-	-	1.56 ± 0.00	3.13 ± 0.00
S. typhimurium	-	-	3.13 ± 0.00	0.39 ± 0.00

Values are expressed as mean ± standard deviation of three replicates. "-": no bacterial growth at all tested concentrations; ">9000.00": more than 9000.00 µg/mL required to inhibit the growth of tested bacteria.

Minimum Bactericidal Concentration (MBC)

From Table 4, EEP was shown to exhibit bactericidal activity against *S. aureus* and *S. mutans* with an MBC value of 4500 µg/mL. However, EEP exhibited bacteriostatic activity against *E. faecalis* (9000 µg/mL), *E. faecium* (9000 µg/mL), and *M. luteus* (280 µg/mL) since the MBC values were higher than the MIC values of the respective bacteria as shown in Table 3. It is also worth noting that WEP exhibited bacteriostatic effects against *S. mutans* (9000 µg/mL).

Table 4. The minimum bactericidal concentration (MBC) of ethanolic extract propolis (EEP) and water extract propolis (WEP) against selected pathogenic bacteria

Bacteria	Concentration (µg/mL)			
	EEP	WEP	Gentamicin[1]	Ceftriaxone[2]
Gram-positive bacteria				
B. subtilis	>9000.00 ± 0.00	>9000.00 ± 0.00	1.56 ± 0.00	0.78 ± 0.00
E. faecalis	9000.00 ± 0.00	-	25.00 ± 0.00	12.50 ± 0.00
E. faecium	9000.00 ± 0.00	-	6.25 ± 0.00	3.13 ± 0.00
M. luteus	280.00 ± 0.00	-	1.56 ± 0.00	1.56 ± 0.00
S. aureus	4500.00 ± 0.00	-	0.10 ± 0.00	6.25 ± 0.00
S. mutans	4500.00 ± 0.00	9000.00 ± 0.00	0.11 ± 0.00	12.50 ± 0.00
Gram-negative bacteria				
E. coli	-	>9000.00 ± 0.00	0.39 ± 0.00	0.10 ± 0.00
K. pneumoniae	-	-	12.50 ± 0.00	50.00 ± 0.00
P. aeruginosa	-	-	1.56 ± 0.00	3.13 ± 0.00
S. typhimurium	-	-	3.13 ± 0.00	0.39 ± 0.00

Values are expressed as mean ± standard deviation of three replicates. "-": no bacterial growth at all tested concentrations. ">9000.00": more than 9000.00 µg/mL required to kill the tested bacteria.

DISCUSSION

The biological characteristics of stingless bee propolis are mainly attributed to its complex phytochemical compositions that are highly variable depending on several factors including geographical region, botanical origin, bee species, and solvents used for extraction (Abdelrazeg *et al*., 2020; Magnavacca *et al*., 2022). Organic solvents such as ethanol and methanol are frequently used in propolis extraction due to their ability to dissolve less polar compounds such as flavonoids, phenolic acids, and terpenoids (Mokhtar *et al*., 2019). On the contrary, water is a polar solvent, and has a lower ability to solubilize less polar compounds compared to organic solvents (Zainal *et al*., 2021). Therefore, previous studies have predominantly focused on investigating the chemical constituents and biological activities of the ethanolic extract of propolis, while water extract has been given less attention. Nevertheless, there is an increasing interest in exploring the potential applications of water extract propolis, particularly in the pharmaceutical and healthcare industries, owing to its favorable properties such as biocompatibility, safer for human consumption, and eco-friendliness (Kubiliene *et al*., 2018). Hence, the present study was carried out to investigate the phytochemical composition and antibacterial activity of both ethanolic extract propolis (EEP) and water extract propolis (WEP) obtained from *H. itama*, which is one of the most commonly reared stingless bee species in Malaysia. Comparative analysis of the two types of extracts may provide valuable insights into their respective potential applications in diverse fields.

In the present study, GC-MS analysis was conducted to determine the chemical profiles of the EEP and WEP of *H. itama*. At least four volatile compounds were identified in EEP, consisting of an organosilicon (hexamethylcyclotrisiloxane), a carbonyl group (2-hydroxy-2-cyclopenten-1-one), a ketone (2-coumaranone), and a silicon ester (diethyl bis(trimethylsilyl) ester silicic acid). The WEP contained an aromatic compound (2-(acetoxymethyl)-3-(methoxycarbonyl)biphenylene) and an organosilicon (hexamethylcyclotrisiloxane). Hexamethylcyclotrisiloxane was identified as a major compound in several medicinal plants, such as *Glochidion candolleanum*, *Olea europaea,* and *Bauhinia acuminata*, as well as two moss species, namely *Cinclidotus fontinaloides* and *Palustriella commutate*, which exhibited potent antibacterial activity against *S. aureus*, *B. subtilis*, *E. coli*, and *P. aeruginosa (*Mostafa *et al*., 2011; Krishna *et al*., 2015; Yayintas *et al*., 2017; Balachandar *et al*., 2022). Another compound, 2-coumaranone, was found to be an effective nematicidal against *Bursaphelenchus xylophilus*, a pine wood nematode that causes pine wilt disease (Sun *et al*., 2022). On the other hand, diethyl bis(trimethylsilyl) ester silicic acid has been shown to potentially enhance the antibacterial activity of *Mucuna pruriens* seeds, even at low concentrations (Kumar & Rajeshkumar, 2017).

Various studies have reported the identification of diverse chemical compounds from different stingless bee propolis using the GC-MS technique. Some of the major compounds identified include flavonoids, terpenoids, phenolics, hydrocarbons, aldehydes, steroids, and fatty acids (Ibrahim *et al*., 2018; Nazir *et al*., 2018; Mohamed *et al*., 2020; Salleh *et al*., 2021). However, in the current study, the GC-MS profiles of both EEP and WEP did not coincide with any known propolis type and demonstrated the lack of secondary metabolites previously found in propolis. The variability of compounds found in stingless bee propolis may be ascribed to several factors, such as differences in geographical location, season, and plant sources visited by the bees (Popova *et al*., 2021). Additionally, differences in the extraction and analytical methods used in each study could also contribute to the disparities observed in the identification of compounds (Kubiliene *et al*., 2015; Pobiega *et al*., 2019; Zainal *et al*., 2021; Kasote *et al*., 2022). It is also noteworthy, that GC-MS analysis of the polar constituents, including flavonoids, phenolic acids, and their esters, present in propolis requires silylation to increase their volatility (Popova *et al*., 2010). However, the current study did not perform this step, which might contribute to the discrepancy in the results obtained.

Findings from this study also indicated that *H. itama* propolis extracts were more effective against Gram-positive than Gram-negative bacteria. This finding corroborated previous reports that propolis extracts were more active against Gram-positive bacteria (Ibrahim *et al*., 2016; Akhir *et al*., 2018; Almuhayawi *et al*., 2020). The difference in sensitivity between Gram-positive and Gram-negative bacteria can largely be attributed to the distinct characteristics of bacterial cell membranes (Breijyeh *et al*., 2020). Gram-negative bacteria tend to be more resistant to antimicrobial agents due to the presence of additional protection provided by the outer membrane that consists of phospholipids and lipopolysaccharides (Prajapati *et al*., 2021). This permeability barrier that is absent in Gram-positive bacteria offers an intrinsic mechanism for protection against foreign substances (Breijyeh *et al*., 2020). The hydrolytic enzymes produced in the outer membrane protein structure of the Gram-negative bacteria can break down the active ingredients of propolis, thus inhibiting its antibacterial action (Sforcin, 2016; Almuhayawi, 2020).

The higher antibacterial activity of EEP shown in this study could be attributed to the polarity of ethanol and its capability to extract more active compounds such as phenolic acid and flavonoids. Studies have shown that the antibacterial activity of propolis corresponds to its polyphenolic compounds such as phenolic acid and flavonoids (Abdelrazeg *et al*., 2020). Furthermore, Adli *et al*. (2022) demonstrated that the EEP of *H. itama* possessed higher total phenolic and flavonoid contents compared to WEP. Similar findings were reported by Kubiliene *et al*. (2015) and Abdullah *et al*. (2019) who indicated that propolis extracted using alcohol and methanol yielded better antimicrobial activity compared to water extract propolis. Additionally, it has been documented that the synergistic activity between various active compounds particularly flavonoids and phenolic acid contributes to the complex antimicrobial activity of propolis (Scazzocchio *et al*., 2006; Jug *et al*., 2014; Lujan *et al*., 2018).

CONCLUSION

This study demonstrated the importance of extraction solvents in obtaining active compounds that contribute to the antibacterial properties of propolis. The results indicated that the EEP of *H. itama* displayed greater antibacterial activity than WEP with higher efficacy against Gram-positive than Gram-negative bacteria. GC-MS analysis further revealed the presence of several volatile compounds, suggesting their potential contribution to the antibacterial activity of EEP. Overall, these results suggested that the EEP of *H. itama* holds promise as a source of antibacterial agent and warrants further investigation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Abdelrazeg, S., Hussin, H., Salih, M., & Shaharuddin, B. (2020). Propolis composition and applications in medicine and health. *International Medical Journal, 25*(3), 1505–1542.

Abdullah, N.A., Ja'afar, F., Yasin, H.M., Taha, H., Petalcorin, M.I., Mamit, M.H., Kusrini, E. and Usman, A. 2019. Physicochemical analyses, antioxidant, antibacterial, and toxicity of propolis particles produced by stingless bee *Heterotrigona itama* found in Brunei Darussalam. Heliyon, 5(9): e02476. https://doi.org/10.1016/j.heliyon.2019.e02476

Adli, M.A., Zohdi, R.M., Othman, N.A., Amin, N.S.M., Mukhtar, S.M., Eshak, Z., Hazmi, I.R. & Jahrudin, D.H.J. 2022. Determination of antioxidant activity, total phenolic and flavonoid contents of Malaysian stingless bee propolis extracts. Journal of Sustainability Science and Management, 17(12): 133-144. https://doi.org/10.46754/jssm.2022.12.012

Akhir, R.A.M., Bakar, M.F.A. & Sanusi, S.B. 2018. Antioxidant and antimicrobial potential of stingless bee (*Heterotrigona itama*) by-products. Journal of Advanced Research in Fluid Mechanics and Thermal Sciences, 42(1): 72-79.

Al-Ani, I., Zimmermann, S., Reichling, J., & Wink, M. 2018. Antimicrobial activities of European propolis collected from various geographic origins slone and in combination with antibiotics. Medicines, 5(1): 2. https://doi.org/10.3390/medicines5010002

Al-Hatamleh, M.A.I., Boer, J.C., Wilson, K.L., Plebanski, M., Mohamud, R. & Mustafa, M.Z. 2020. Antioxidant-based medicinal properties of stingless bee products: Recent progress and future directions. Biomolecules, 10(6): 923. https://doi.org/10.3390/biom10060923

Almuhayawi, M.S. 2020. Propolis as a novel antibacterial agent. Saudi Journal of Biological Sciences, 27(11): 3079–3086. https://doi.org/10.1016/j.sjbs.2020.09.016

Anjum, S.I., Ullah, A., Khan, K.A., Attaullah, M., Khan, H., Ali, H., Bashir, M.A., Tahir, M., Ansari, M.J., Ghramh, H.A., Adgaba, N. & Dash, C.K. 2019. Composition and functional properties of propolis (bee glue): A review. Saudi Journal of Biological Sciences, 26(7): 1695–1703. https://doi.org/10.1016/j.sjbs.2018.08.013

Asma, S.S., Adam, N.A., Najwa, M.Y.S., Asilah, N., Syukri, T.S. & Asiah, W.N. 2019. Foraging behavior of stingless bee (*Geniotrigona thoracica* and *Heterotrigona itama*) on star fruit trees (*Averrhoa carambola* L.). IOP Conference Series: Earth and Environmental Science, 383: 012011. https://doi.org/10.1088/1755-1315/383/1/012011

Azmi, W.A., Wan Sembok, W.Z., Mohd Nasaruddin, S.N., Azli, N.S., Mohd Hatta, M.F. & Tg Muhammad, T.N. 2022. Evaluation of native stingless bee species (*Heterotrigona itama* and *Geniotrigona thoracica*) for pollination efficiency on Melon Manis Terengganu. Malaysian Applied Biology, 51(5): 229–235. https://doi.org/10.55230/mabjournal.v51i5.2360

Azmi, W.A., Ghazi, R. & Nasharuddin, I.S. 2019. Morphological, Nest Architecture and Colony Characteristics of Stingless Bees (Hymenoptera; Apidae; Meliponini) from Tasik Kenyir, Terengganu. In: Greater Kenyir Landscapes. M. Abdullah, A. Mohammad, M. Nor Zalipah, M. Safiih Lola (Eds.). Springer, Cham. https://doi.org/10.1007/978-3-319-92264-5_11

Balachandar, R., Navaneethan, R., Biruntha, M., Kumar, K.K.A., Govarthanan, M. & Karmegam, N. 2022. Antibacterial activity of silver nanoparticles phytosynthesized from *Glochidion candolleanum* leaves. Materials Letters, 311: 131572. https://doi.org/10.1016/j.matlet.2021.131572

Breijyeh, Z., Jubeh, B. & Karaman, R. 2020. Resistance of Gram-positive bacteria to current antibacterial agents and overcoming approaches. Molecules, 25(12): 2888. https://doi.org/10.3390/molecules25122888

Clinical And Laboratory Standards Institute. (2018). *M100 Performance Standards for Antimicrobial Susceptibility Testing (28th ed.)*.

Devequi-Nunes, D., Machado, B.A.S., Barreto, G.D.A., Rebouças Silva, J., da Silva, D.F., da Rocha, J.L.C., Brandão, H.N., Borges, V.M. & Umsza-Guez, M.A. 2018. Chemical characterization and biological activity of six different extracts of propolis through conventional methods and supercritical extraction. PLoS ONE, 13(12): e0207676. https://doi.org/10.1371/journal.pone.0207676

Fahimee, J., Badrulisham, A.S., Zulidzham, M.S., Reward, N.F., Muzammil, N., Jajuli, R., Md-Zain, B.M. & Yaakop. S. 2021. Metabarcoding in Diet Assessment of *Heterotrigona itama* Based on trnL Marker towards Domestication Program. Insects, 12(3): 205. https://doi.org/10.3390/insects12030205

Ibrahim, N., Niza, N.F.S.M., Rodi, M.M., Zakaria, A.J., Ismail, Z. & Mohd, K.S. 2016. Chemical and biological analyses of Malaysian stingless bee propolis extracts. Malaysian Journal of Analytical Sciences, 20(2): 413-422.

Ivorra, T., Hauser, M., Low, V.L., Tomberlin, J.K., Azmi, N.A.N., Cammack, J.A. & Heo, C.C. 2020. *Hermetia illucens* and *Hermetia fenestrata* (Diptera: Stratiomyidae) colonization of spoiled stingless bee *Geniotrigona thoracica* (Hymenoptera: Apidae) hives in Malaysia. Insects, 11(11): 737. https://doi.org/10.3390/insects11110737

Jaapar, M.F., Halim, M., Mispan, M.R., Jajuli, R., Saranum, M.M., Zainuddin, M.Y., Ghazi, R. & Abd Ghani, I. 2016. The diversity and abundance of stingless bee (Hymenoptera: Meliponini) in Peninsular Malaysia. Advances in Environmental Biology, 10(2): 1–8.

Jug, M., Končić, M.Z. & Kosalec, I. 2014. Modulation of antioxidant, chelating and antimicrobial activity of poplar chemo-type propolis by extraction procures. LWT - Food Science and Technology, 57(2): 530–537. https://doi.org/10.1016/j.lwt.2014.02.006

Kasote, D., Bankova, V. & Viljoen, A.M. 2022. Propolis: Chemical diversity and challenges in quality control. Phytochemistry Reviews, 21: 1887–1911. https://doi.org/10.1007/s11101-022-09816-1

Kasote, D.M., Pawar, M.V., Gundu, S.S., Bhatia, R., Nandre, V.S., Jagtap, S.D., Mahajan, S.G. & Kulkarni, M.V. 2019. Chemical Profiling, Antioxidant, and Antimicrobial Activities of Indian Stingless Bees Propolis Samples. Journal of Apiculture Research, 58: 617-625. https://doi.org/10.1080/00218839.2019.1584960

Khalifa, S.A.M., Elshafiey, E.H., Shetaia, A.A., El-Wahed, A.A.A., Algethami, A.F., Musharraf, S.G., AlAjmi, M. F., Zhao, C., Masry, S.H.D., Abdel-Daim, M.M., Halabi, M.F., Kai, G., Al Naggar, Y., Bishr, M., Diab, M.A.M., & El-Seedi, H.R. 2021. Overview of bee pollination and its economic value for crop production. Insects, 12(8): 688. https://doi.org/10.3390/insects12080688

Krishna, S.R.A., Hafza, S., Poorna Chandrika, G., Lekhya Priya, C. & Bhaskara Rao, K.V. 2015. Pharmacological properties, phytochemical and GCMS analysis of Bauhinia acuminata Linn. Journal of Chemical and Pharmaceutical Research, 7(4): 372-380.

Kubiliene, L., Jekabsone, A., Zilius, M., Trumbeckaite, S., Simanaviciute, D., Gerbutaviciene, R. & Majiene, D. 2018. Comparison of aqueous, polyethylene glycol-aqueous and ethanolic propolis extracts: Antioxidant and mitochondria modulating properties. BMC Complementary and Alternative Medicine, 18: 165). https://doi.org/10.1186/s12906-018-2234-5

Kubiliene, L., Laugaliene, V., Pavilonis, A., Maruska, A., Majiene, D., Barcauskaite, K., Kubilius, R., Kasparaviciene, G. & Savickas, A. 2015. Alternative preparation of propolis extracts: Comparison of their composition and biological activities. BMC Complementary and Alternative Medicine, 15: 156. https://doi.org/10.1186/s12906-015-0677-5

Kumar, V.S. & Rajeshkumar, S. 2017. Anti-inflammatory, antioxidant, antibacterial effect and phytochemical analysis of *Mucuna pruriens* seed extract. International Journal of ChemTech Research, 10(1): 273–283.

Kustiawan, P.M., Yanti, E.N., Nisa, K., Zulfa, A.F. & Batistuta, M.A. 2023. Bioactivity of *Heterotrigona itama* propolis as anti-inflammatory: A review. Biointerface Research in Applied Chemistry, 13(4): 326. https://doi.org/10.33263/BRIAC134.326

Li, Y.R., Wang, Z.W., Yu, Z.R. & Corlett, R.T. 2021. Species diversity, morphometrics, and nesting biology of Chinese stingless bees (Hymenoptera, Apidae, Meliponini). Apidologie, 52: 1239-1255. https://doi.org/10.1007/s13592-021-00899-x

Luján, M.de R.M., Reséndez, A.M., Barrón, G.S.G., Carrillo, J.L.R., Inungaray, M.L.C. 2018. Antibacterial activity and phenolic content of propolis extracts obtained by different extraction methods. Nova Scientia, 10(20): 397-412. https://doi.org/10.21640/ns.v10i20.1392

Magnavacca, A., Sangiovanni, E., Racagni, G. & Dell'Agli, M. 2022. The antiviral and immunomodulatory activities of propolis: An update and future perspectives for respiratory diseases. Medicinal Research Reviews, 42(2): 897–945. https://doi.org/10.1002/med.21866

Mokhtar, S.U., Hooi, H.S., Lene, D.T.T. & Jayaraman, S. 2019. Comparison of total phenolic and flavonoids contents in Malaysian propolis extract with two different extraction solvents. International Journal of Engineering Technology and Sciences, 6(2): 1–11. https://doi.org/10.15282/IJETS.V6I2.2577

Mostafa AA, Al-Rahmah AN, Abdel-Megeed A. Evaluation of some plant extracts for their antifungal and antiaflatoxigenic activities. J Med Plants Res, 2011; 5(17):4231-4238.

Pasupuleti, V.R., Sammugam, L., Ramesh, N. & Gan, S.H. 2017. Honey, propolis, and royal jelly: A comprehensive review of their biological actions and health benefits. Oxidative Medicine and Cellular Longevity, 2017: 1259510. https://doi.org/10.1155/2017/1259510

Pobiega, K., Kraśniewska, K., Derewiaka, D. & Gniewosz, M. 2019. Comparison of the antimicrobial activity of propolis extracts obtained by means of various extraction methods. Journal of Food Science and Technology, 56(12): 5386–5395. https://doi.org/10.1007/s13197-019-04009-9

Popova, M., Trusheva, B. & Bankova, V. 2021. Propolis of stingless bees: A phytochemist's guide through the jungle of tropical biodiversity. Phytomedicine, 86: 153098. https://doi.org/10.1016/j.phymed.2019.153098

Prajapati, S.K., Mishra, G., Malaiya, A., Jain, A., Mody, N. & Raichur, A.M. 2021. Antimicrobial application potential of phytoconstituents from turmeric and garlic. In: Bioactive Natural Products for Pharmaceutical Applications. D. Pal. & A.K. Nayak (Eds.). pp. 409-435. https://doi.org/10.1007/978-3-030-54027-2_12

Salleh, S.N.A.S., Hanapiah, N.A.M., Ahmad, H., Johari, W.L.W., Osman, N.H. & Mamat, M.R. 2021. Determination of total phenolics, flavonoids, and antioxidant activity and GC-MS analysis of Malaysian stingless bee propolis water extracts. Scientifica, 2021: 3789351. https://doi.org/10.1155/2021/3789351

Salleh, S.N.A.S., Johari, W.L.W. & Hanapiah, N. A.M. 2022. A comprehensive review on chemical compounds, biological actions and potential health benefits of stingless bee propolis. Sains Malaysiana, 51(3): 733–745. https://doi.org/10.17576/jsm-2022-5103-08

Sarker, S.D., Nahar, L. & Kumarasamy, Y. 2007. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. Methods, 42(4): 321–324. https://doi.org/10.1016/j.ymeth.2007.01.006

Scazzocchio, F., D'Auriaa, F.D., Alessandrinia, D. & Pantanella, F. 2006. Multifactorial aspects of antimicrobial activity of propolis. Microbiology Research, 161(4): 327-333. https://doi.org/10.1016/j.micres.2005.12.003

Sforcin, J.M. 2016. Biological properties and therapeutic applications of propolis. Phytotherapy Research, 30(6): 894–905. https://doi.org/10.1002/ptr.5605

Souza, E.C.A., Menezes, C. & Flach, A. 2021. Stingless bee honey (Hymenoptera, Apidae, Meliponini): A review of quality control, chemical profile, and biological potential. Apidologie, 52: 113–132. https://doi.org/10.1007/s13592-020-00802-0

Sun, Y., Wang, C., Du, G., Deng, W., Yang, H., Li, R., Xu, Q. & Guo, Q. 2022. Two nematicidal compounds from *Lysinimonas* M4 against the pine wood nematode, *Bursaphelenchus xylophilus*. Forests, 13: 1191. https://doi.org/10.3390/f13081191

Touzani, S., Embaslat, W., Imtara, H., Kmail, A., Kadan, S., Zaid, H., ElArabi, I., Badiaa, L. & Saad, B. 2019. In vitro evaluation of the potential use of propolis as a multitarget therapeutic product: Physicochemical properties, chemical composition, and immunomodulatory, antibacterial, and anticancer properties. BioMed research international, 2019: 4836378. https://doi.org/10.1155/2019/4836378

Yayintas, O.T., Alpaslan, D., Yuceer, Y.K., Yilmaz, S. & Sahiner, N. 2017. Chemical composition, antimicrobial, antioxidant and anthocyanin activities of mosses (*Cinclidotus fontinaloides* (Hedw.) P.Beauv. and *Palustriella commutata* (Hedw.) Ochyra) gathered from Turkey. Natural Product Research, 6419: 1–5. https://doi.org/10.1080/14786419.2016.1277355

Zainal, W.N.H.W., Azian, N.A.A.M., Albar, S.S. & Rusli, A.S. 2021. Effects of extraction method, solvent and time on the bioactive compounds and antioxidant activity of *Tetrigona apicalis* Malaysian propolis. Journal of Apicultural Research, 61(2): 264-270. https://doi.org/10.1080/00218839.2021.1930958